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Ligand-modulated conformational switching in a fully synthetic membrane-bound receptor

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Abstract

Signal transduction through G protein-coupled receptors (GPCRs) involves binding to signalling molecules at the cell surface, which leads to global changes in molecular conformation that are communicated through the membrane. Artificial mechanisms for communication involving ligand binding and global conformational switching have been demonstrated so far only in the solution phase. Here we report a membrane-bound synthetic receptor that responds to binding of a ligand by undergoing a conformational change that is propagated over several nanometres, deep into the phospholipid bilayer. Our design uses a helical foldamer core, with structural features borrowed from a class of membrane-active fungal antibiotics, ligated to a water-compatible, metal-centred binding site and a conformationally-responsive fluorophore. Using the fluorophore as a remote reporter of conformational change, we find that binding of specific carboxylate ligands to a Cu(II) cofactor at the binding site perturbs the foldamer's global conformation, mimicking the conformational response of a GPCR to ligand binding.

Controlling the transmission of biochemical information across phospholipid bilayers is an enormous opportunity in synthetic biology. Artificial signal transduction would allow orthogonal signalling pathways to be introduced into cells, "short-circuiting" their signalling networks, or would facilitate communication between compartments[1] in artificial tissues.[2] Cellular signal transduction is largely mediated by three classes of membrane proteins: tyrosine kinase receptors, ligand-gated channels and (most abundantly) G protein-coupled receptors (GPCRs). Efforts have been made to replicate the mode of action of first two,[3-6] but mimics of GPCRs, which offer the greatest scope for use in synthetic biology, have proved elusive. While many structural details of GPCR-mediated signal transduction are poorly understood,[7] a general mechanism is clear.[8,9] GPCRs, which comprise seven transmembrane helices, adopt a number of interchanging conformational states in the membrane.[10,11] Selective binding of an appropriate signalling ligand at a site exposed to the extracellular medium induces a local conformational preference in the tertiary structure of a receptor, which propagates through the GPCR and across the bilayer to the cytosolic terminus >4 nm distant.[12] This new conformational state, which predominates over the others, initiates the signalling cascade. Synthetic molecules have been designed that have functional analogy with ligand-activated GPCRs like the β2 adrenoreceptor,[1] but they work only in organic solvents.[13-16]

Results and Discussion

Design of the receptor mimic. We set out to design and construct a synthetic molecular structure exhibiting the 'minimal' essential design features required for a functional mimic of a ligand-activated GPCR. The key aspects of the design are shown in Fig. 1. It has the following features: (i) a hydrophobic scaffold (Fig. 1b, shown in grey) that inserts into a phospholipid bilayer and has molecular dimensions commensurate with bilayer width; (ii) two interconverting conformational states that are perturbed by binding of a chiral ligand to a binding site; (iii) a binding pocket (Fig. 1b, green) with a metal(II) ion cofactor that allows strong, reversible, and shape-selective binding of aqueous ligands (Fig. 1b, red) at the bilayer interface, even in the presence of water; (iv) a remote group that gives a detectable spectroscopic response to changes in conformational state (Fig. 1b, blue).

As the molecular scaffold forming the core of the receptor, we chose a structural motif characteristic of peptaibols,[17] fungal antibiotics that insert into and permeabilise membranes. Peptaibols are rich in the quaternary amino acid α-aminoisobutyric acid (Aib), which induces them to fold into helical conformations with hydrophobic character. Synthetic oligomers built solely from Aib fold into 3_{10} helices (Figure 1a, "Relay"),[18] with decamers being sufficiently long to span a bilayer.[19] In solution, these "foldamers"[20] populate almost exclusively[21] two rapidly exchanging conformational states of left or right handed helical screw sense, with the relative population of these two states being remarkably sensitive to chiral influences.[22-25] A chiral ligand covalently[15] or non-covalently[16] bound to one terminus of an Aib foldamer will propagate its conformational influence through the entire foldamer length.[26] In this way end-to-end transmission of binding information over multi-nanometre distances has been accomplished through Aib foldamers dissolved in organic solvents.[15,27,28]

Recently, covalently controlled conformational switching over multi-nanometre distances within membrane-bound Aib foldamers was demonstrated using a light-switchable molecule.[29]

The ligand binding pocket was constructed from a cationic metal(II) bis(2-quinolylmethyl)(2-pyridylmethyl)amine (BQPA) complex (Fig. 1a, "Input").[30] Complexation of a chiral guest (such as a carboxylate, Fig. 2a) in acetonitrile induces the achiral BQPA-metal complex to adopt preferentially one of two mirror-image (*M* or *P*) propeller-shaped conformations.[31] To validate foldamer-linked Cu-BQPA as a conformationally responsive binding site, incremental amounts of enantiomeric ligands Boc-L-Pro (L-**2a**) or Boc-D-Pro (D-**2a**) in CH_3CN were added to Cu-**3** bearing an N-terminal copper(II) BQPA complex (Supplementary Fig. 4.11). Equal and opposite changes in the Cotton effect at 239 nm were observed, confirming tight binding (K_d < 1 μM) of these prototypical chiral ligands to the metal ion and induction of chirality in the BQPA binding pocket (Fig. 2f).

Pyrene fluorescence was used to detect the conformational response of the receptor mimic when embedded in a vesicle membrane. Other analytical techniques typically used for conformational analysis of foldamers in solution[14-16] were found to be ill-suited to the membrane phase and/or Cu(II) ions. Pyrene has a monomer fluorescence emission at 378 nm, and depending on their proximity and relative orientation, a pair of pyrene fluorophores may form an excimer that emits at 450 nm.[13] A ratiometric fluorescent probe of conformation was synthesised (Fig. 1a, "Output") by incorporating two pyrene units into a chiral diamine, which was ligated to the terminus of an Aib oligomer. The two helical screw-sense conformations of the Aib foldamer were expected to induce diastereoisomeric conformations in the fluorophore (Figure 1b, in blue), each with a characteristic ratio of excimer to monomer emission (E/M ratio). Fluorescence spectroscopy of a series of Aib foldamers bearing covalently linked N-terminal chiral residues showed a clear dependence of the E/M ratio on the ratio of screw-sense populations in solution (see Supplementary Fig. 4.10), with an increase in the proportion of *P* helix causing an increase in E/M.

Function of the receptor mimic in solution. The synthesis of receptor **1** builds a structure that has these three components - a binding site, a signal relay and a fluorescent reporter (Fig. 1). After adding the copper(II) cofactor, the resulting complex Cu-**1** was treated with a pair of enantiomeric carboxylates in acetonitrile solution (Fig. 2a,b). Adding incremental amounts of Boc-L-Pro (L-**2a**) and Boc-D-Pro (D-**2a**) in CH_3CN caused changes in both the CD and fluorescence spectra (Fig. 2c-e) not seen in a control Aib foldamer **4** lacking the Cu(BQPA) binding pocket (Fig. 2g and Supplementary Fig. 4.11). Crucially, the enantiomers L-**2a** and D-**2a** gave opposite responses; L-**2a** induced positive ellipticity at 239 nm, an increase in excimer emission and an increase in E/M ratio (from 1.65 to 1.82), whereas D-**2a** induced negative ellipticity, a decrease in excimer emission and a decrease in E/M (from 1.65 to 1.42). These parallel spectral changes indicate firstly that a local chiral preference is induced in the BQPA binding site (giving rise to the Cotton effect at 239 nm), and secondly that the conformational preference is transmitted through the helical oligomer to the remote C-terminal fluorophore (inducing a change in excimer fluorescence that produces a change

in E/M ratio). Analysis of the CD and excimer emission data using a 1:1 binding model showed tight binding to **1** in CH_3CN, providing K_d = 1 μM for D-**2a** and K_d = 1.7 μM for L-**2a**.

Function of the receptor mimic in the membrane. With the receptor mimic Cu-**1** functioning in an organic solvent, we explored its behaviour in the membrane phase. Vesicles were made from the phospholipid 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), which at 25 °C gives fluid bilayers[32] that allowed Cu-**1** the conformational freedom to respond to ligand binding. DOPC bilayers also have a hydrophobic thickness of 2.88 nm[32] commensurate with the 2.6 nm estimated hydrophobic length of Cu-**1**. An aliquot of Cu-**1** in acetonitrile was added to a suspension of large unilamellar vesicles (LUVs, 800 nm diameter, at pH 7.4) to give vesicles with 0.4 mol% Cu-**1**, a low loading that minimised inter-receptor contacts. A rapid >100-fold increase in fluorescence occurred (Fig. 3b,c), consistent with insertion of a pyrene moiety into a bilayer.[33] This increase in emission stabilised after 25 minutes, leading to steady value of E/M = 1.25 (Fig. 3c). A similar value for E/M was found for membrane loadings from 0.2 to 1 mol% (Supplementary Fig. 6.3), suggesting that at these loadings intermolecular excimer formation due to aggregation is not significant. Incorporation of Cu-**1** into bilayers was confirmed by fluorescence microscopy (Fig. 3d), which showed pyrene excimer fluorescence localised in the LUVs. The fluorescence emission from Cu-**1** in buffer alone was much weaker than the emission from membrane-embedded Cu-**1** (see Supplementary Information Section 6.5.1), allowing the effect of any non-inserted Cu-**1** to be disregarded.

The conformational responsiveness of membrane-bound Cu-**1** was explored by adding carboxylate ligands to the suspension of vesicles. As in isotropic solution, the addition of chiral ligands L-**2a** and D-**2a** (Fig. 4a) to Cu-**1** in a bilayer produced changes in its fluorescence spectrum, although much higher concentrations of ligand (100 times greater) were required to achieve a similar level of response. Intriguingly the membrane environment reversed the conformational response of the pyrene probe, indicating that an increase in E/M now corresponds to an increase in the proportion of *M* helix. This reversal of fluorescent response also occurred for covalently controlled foldamers in bilayers (Supplementary Fig. 5.1) whereas the CD titration of Cu-**3** in bilayers with L-**2a**, which gave a positive signal at 239 nm (Supplementary Fig. 6.6.1), showed that the induction of screw sense at the BQPA binding site occurred with the same sense as in solution (Fig. 2f). Since opposite enantiomers of **2a** still induced opposite responses in Cu-**1** (L-**2a** decreasing and D-**2a** increasing the excimer emission intensity and the E/M ratio), a generalised ligand-induced change in bilayer properties could be ruled out as the origin of the fluorescence modulation. Additionally, homologous foldamers with shorter Aib chains (Aib_4 in place of Aib_8) showed no fluorescent response, not only confirming that there is no direct interaction between ligand and fluorophore but also suggesting that higher hydrophobicity may promote deeper membrane insertion that is required for function. Fitting the changes in Cu-**1** excimer emission to a 1:1 binding model confirmed that binding of these agonists to membrane-bound Cu-**1** was 100-fold weaker than in CH_3CN solution (L-**2a** gave $K_d \approx 140$ μM; D-**2a** gave $K_d \approx 60$ μM). The half maximal effective concentration (EC_{50}) values, which are commonly used[34] to quantify responses of membrane-bound receptors, were calculated by

fitting the changes in E/M. This provided EC_{50} = 130 ± 30 μM and 70 ± 30 μM for L-**2a** and D-**2a** respectively.

It was not possible to assign with confidence the precise orientation of the foldamer in the bilayer. The ability of the polar carboxylate ions to enter the binding site implies this polar Cu(II)-bearing moiety is exposed to the aqueous environment surrounding the vesicles. Comparing the ratio of monomer emission intensities from the I and III emission bands from the pyrene fluorophore[35] of Cu-**1** in organic solvents to Cu-**1** in a bilayer indicates that the pyrenes are in a polar environment to which water has access. This suggests that the pyrenes lie within the membrane but close to the interface with water, possibly on the other side of the bilayer (foldamers of similar length and structure are capable of spanning the bilayer[19]).

Other carboxylates appeared to bind within the pocket, such as unprotected L-proline (EC_{50} ~ 130 μM), but these agonists had lower efficacy and only induced very weak conformational responses in membrane-embedded Cu-**1**. Glutamate and aspartame induced no change in fluorescence, although another Boc-protected amino acid, Boc-L-Leu L-**2c**, induced a decrease in E/M (EC_{50} = 40 ± 20 μM), albeit more weakly than L-**2a.** Binding seemed to translate into a remote conformational response only with carboxylate ligands containing an amide-like carbonyl group, probably through hydrogen bonding to an adjacent Aib residue in the helical oligomer (see Fig. 1a and Supplementary Fig. 6.7.4). With this in mind, Leu-enkephalin (Tyr-Gly-Gly-Phe-Leu, **2d**), an endogenous opioid peptide neurotransmitter that binds μ- and δ-opioid GPCRs,[36] was tested. Remarkably we found that Leu-enkephalin (EC_{50} = 70 ± 30 μM) induced a powerful response in our receptor mimic, increasing E/M significantly.

Carboxylate ligand binding to membrane-bound Cu-**1** is reversible, and this feature was demonstrated using competitive binding between an "agonist" and an "antagonist". Addition of the "agonist" Leu-enkephalin **2d** resulted in an increase in E/M in Cu-**1**, which could be reversed and then inverted by competitive binding of the "antagonist" L-BocPro L-**2a** (Fig. 4c). Ligand L-**2a** thus behaves in a manner analogous to μ- and δ-opioid GPCR antagonists such as naloxone.[37,38]

Conclusions.

Designed synthetic molecules **1** are far simpler than biological receptors of the GPCR family, yet they display all the characteristics required of a functional, synthetic, 'minimal' receptor. They embed themselves into a membrane, they exhibit selective and competitive binding of certain classes of ligand at a well-defined binding site, they change conformation as a result of ligand binding, and the resulting conformational change is transmitted through the receptor to a site remote from the binding site. Cu-**1** and its congeners offer a rare opportunity to compare the conformational behaviour of a molecule in solution and in the membrane phase, and to probe the effect of the membrane on conformational preference and ligand binding. Receptor **1** is of a length comparable with the thickness of a typical cell membrane, and we are currently exploring the possibility of using its metal complexes to transmit chemical information

through a membrane barrier, with the aim of controlling functional behaviour in artificial vesicles, and ultimately even in living cells.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Data Availability

All relevant data is included as Supplementary Information.

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Author contributions

SJW and JC conceived the project and wrote the paper; FGAL, BAFL, SJW and JC devised the experiments and analysed the data; FGAL and BAFL carried out the experiments.

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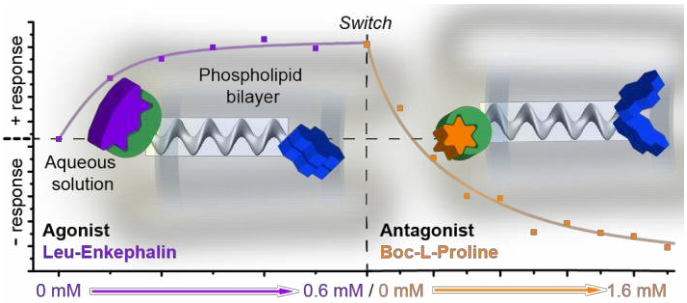
Summary for TOC:



A dynamic foldamer scaffold borrowed from a class of membrane-active fungal antibiotics was ligated to a water-compatible, metal-centred binding site and a conformationally-responsive fluorophore, providing a receptor mimic that inserts into the membrane of artificial vesicles. The binding site selected for specific carboxylate ligands, which induced fluorimetrically-detectable global conformational changes that depended on the structure of the ligand.

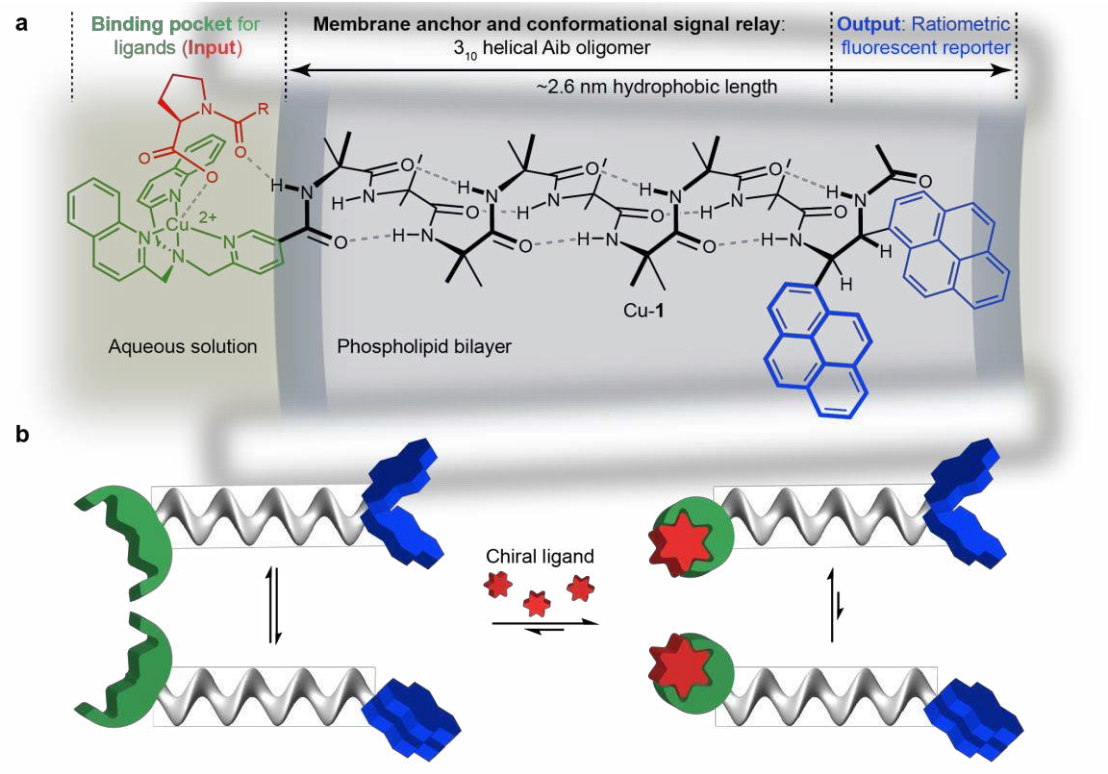


Figure 1: Design and Function of a Synthetic GPCR mimic. The receptor mimic is built from three components: a metal-containing binding site (shown in green), a helical Aib oligomer (shown in black or grey) which allows the receptor to embed into the membrane and relays conformational changes from the binding site to the reporter, and a fluorophore (shown in blue) to provide a spectroscopic indicator of conformational change. **a**, Structure of a synthetic GPCR mimic **1** with copper(II) cofactor binding a carboxylate ligand (shown in red). **b**, Change in equilibrium population distribution of right and left-handed screw-sense conformers in response to reversible binding of a chiral carboxylate ligand (shown in red).

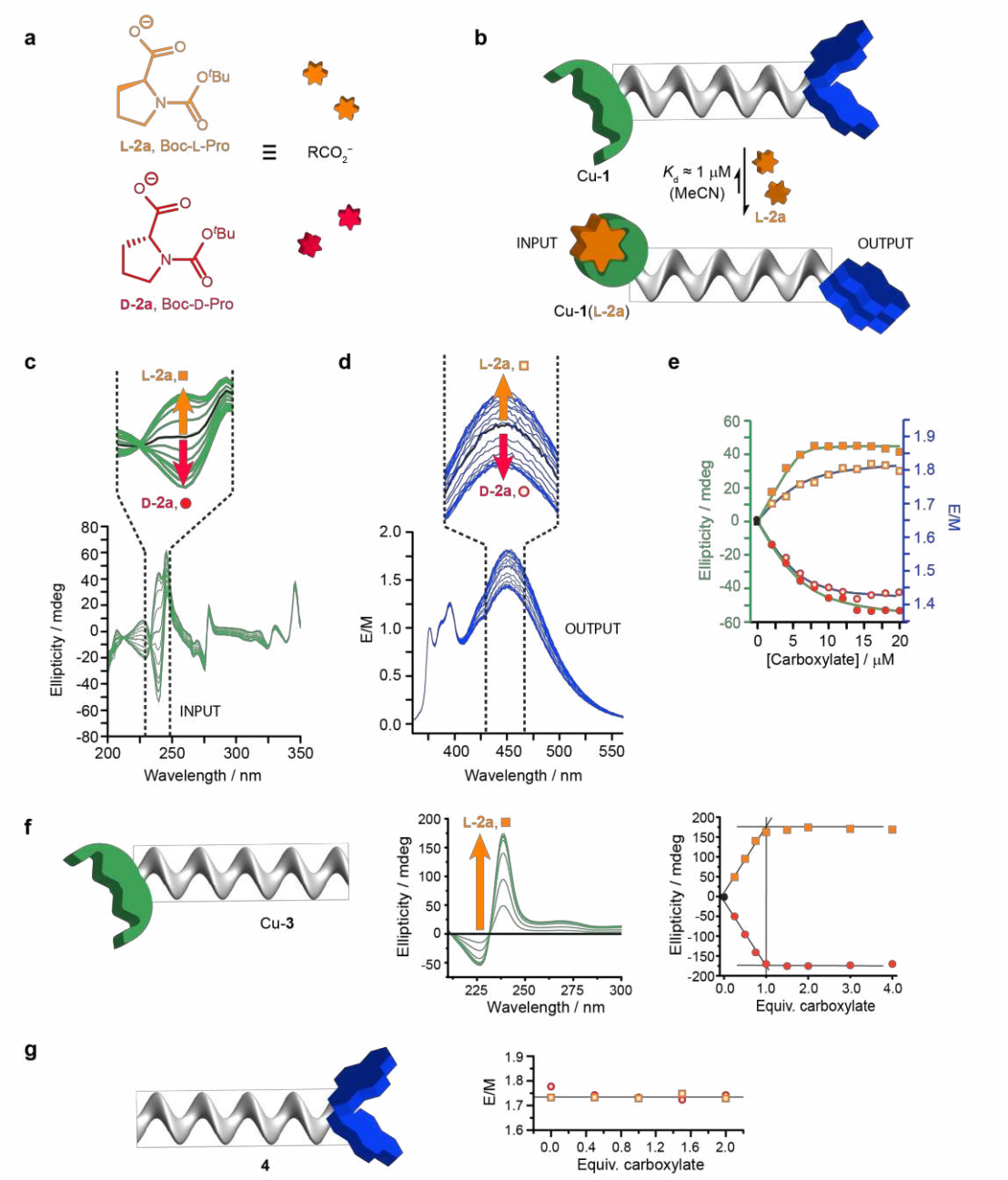


Figure 2: Ligand-induced conformational responses in solution. Binding of enantiomeric carboxylate ligands Boc-L-Pro (L-**2a**, shown in orange) or Boc-D-Pro (D-**2a**, shown in red) induce opposite effects at the remote fluorescent reporter through a conformational relay mechanism that involves an inversion of screw sense in the helical domain of the receptor. **a,** Structures of the chiral carboxylate ligands used in the study. **b**, Tight but reversible complexation of chiral ligand L-**2a** to receptor Cu-**1** in organic solvent, inducing a change in the relative population of conformers of the fluorophore. **c,d**, Corresponding changes in the CD (**c**) and fluorescence (**d**) spectra of Cu-**1** (10 μM in acetonitrile) on addition of L-**2a** (direction of change shown by orange arrow) and D-**2a** (direction of change shown by redarrow). The CD spectrum (**c**) reports a local change in conformation at the binding site, while the fluorescence spectrum (**d**) reports a global change in conformation of the receptor, relayed to the remote fluorophore. **e**, Changes in the E/M ratio (outline markers) and ellipticity at 239 nm (solid markers) upon incremental addition of L-**2a** (orange markers) and D-**2a** (red markers) to Cu-**1**. Curve fits shown for K_d = 1 μM (D-**2a**) and K_d = 1.7 μM (L-**2a**). **f**, Changes in CD spectra upon addition of L-**2a** (shown as a series of spectra, direction of change indicated by orange arrow, and plotted as orange points) and D-**2a** (plotted as red points) to modified receptor Cu-**3** (0.25 mM), which contains a binding site (green) but no fluorescent reporter. **g**, Changes in E/M ratio upon addition of L-**2a** and D-**2a** to helical foldamer

4 (10 μM in acetonitrile), which contains the fluorescent reporter (blue) but no binding site. The lack of response on addition to either L-**2a** (orange markers) or D-**2a** (red markers) indicates that the binding site is required for the remote induction of a fluorescent response.

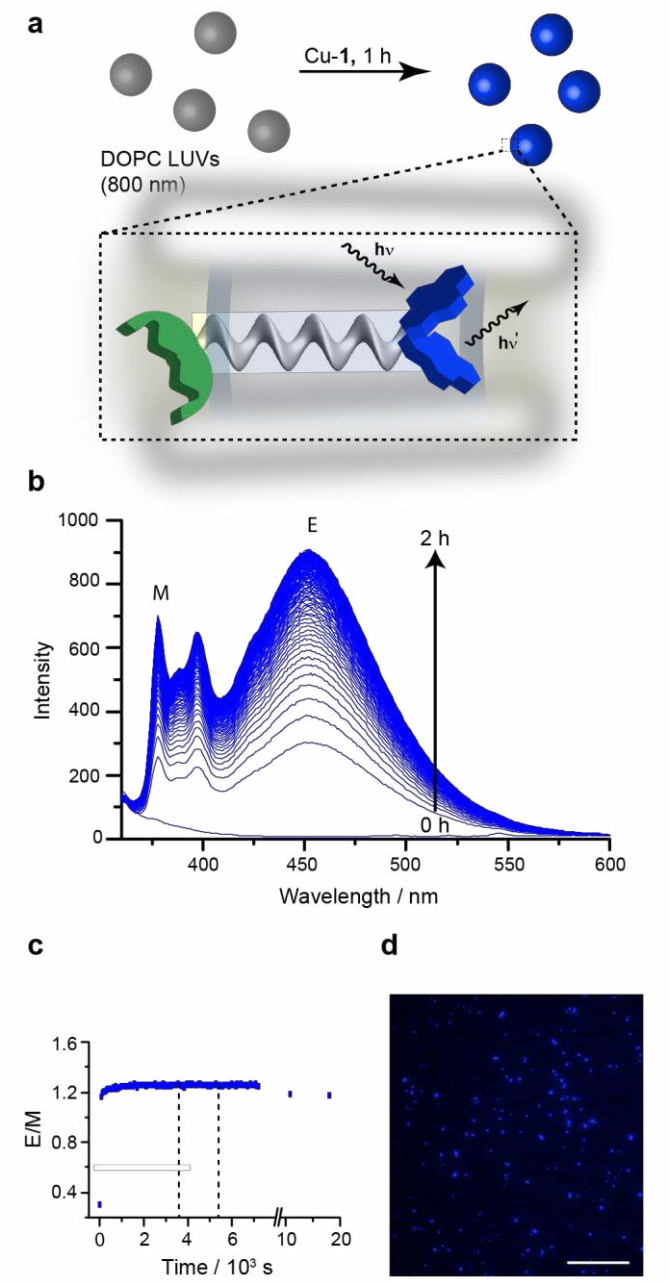


Figure 3: Incorporation of the receptor mimic into the membrane of artificial vesicles. The receptor mimic migrates from solution to the phospholipid bilayer of the vesicles to generate a stable, fluorescent membrane-embedded structure over a period of 1-2 h **a**, Schematic representation of Cu-**1** inserting into the membranes of DOPC LUVs and becoming fluorescent. **b**, Change in fluorescence of Cu-**1** over 2 h after addition to DOPC LUVs, showing in increase in both excimer (E) and monomer (M) emission. **c**, Change in E/M ratio in the fluorescence emission from Cu-**1** after mixing with DOPC LUVs. In binding experiments, ligands were added after the E/M value had stabilised, in the period between 1 and 1.5 h after addition of the receptor to the vesicles, as indicated by dotted lines. **d**, Fluorescence microscopy image of DOPC LUVs after mixing with Cu-**1**, showing fluorescence localised in the vesicle membranes. Scale bar = 100 μm.

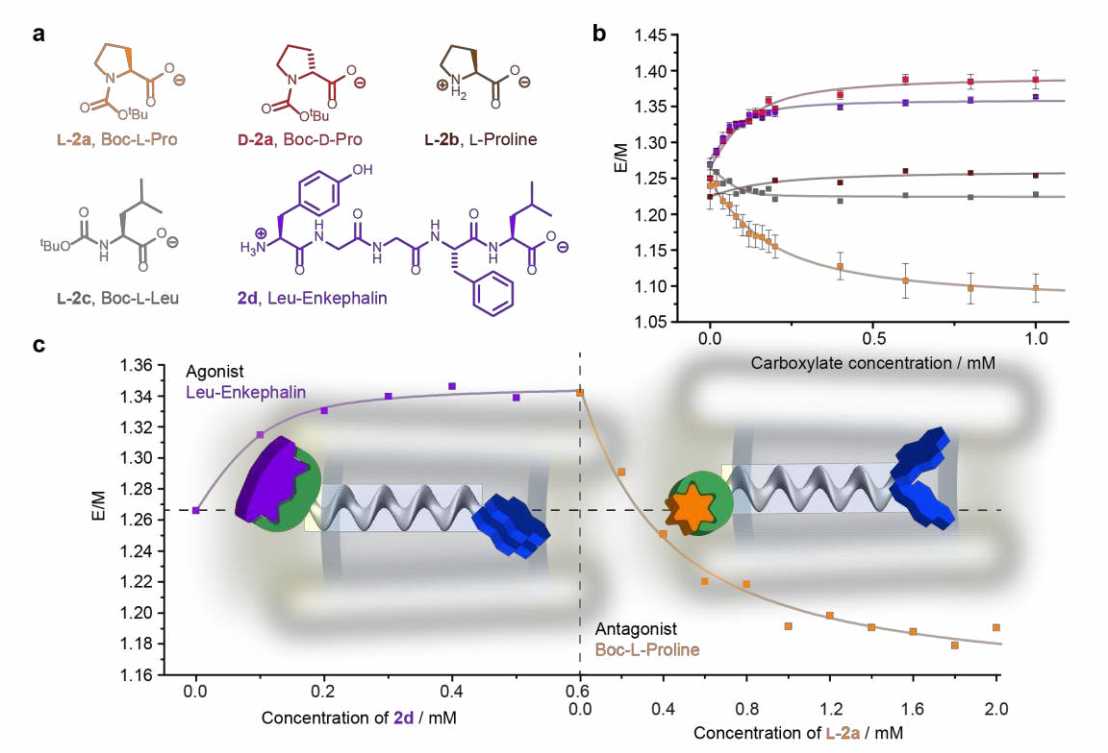


Figure 4: Ligand-induced conformational responses in the membrane phase. Adding carboxylate ligands to the membrane-embedded receptors induces characteristic conformational changes, detected as changes in the excimer to monomer (E/M) emission ratio. **a**, Suite of chiral ligands **2a** – **2d** screened against membrane-bound Cu-**1** (10 μM, 0.4 mol% loading in DOPC vesicles). **b**, Changes in the fluorescence spectra of membrane-bound Cu-**1** after addition of ligands L-**2a**, D-**2a**, L-**2b**, L-**2c**, **2d**, coloured as in **a** (EC_{50} values from curve fits: 144, 98, 138, 55, 72 μM respectively). Indicated errors are the standard deviations of at least three sets of measured data. **c**, Switching between conformation populations of Cu-**1** by addition of "agonist" Leu-enkephalin **2d** (shown in purple, curve fit EC_{50} = 72 μM) then "antagonist" Boc-L-Pro L-**2a** (shown in orange, competition with **2d** gives EC_{50} = 360 μM).